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Biotechnology for Near Real-Time Predictive Toxicology for Warfighter Protection

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ABSTRACT

An increasingly important issue in force protection is the toxicology associated with toxic chemical and mixture exposure at uncharacterized deployed sites. Current methods for determining or monitoring toxic exposures to the warfighter in their working or living environment are not adequate to prevent serious health effects. Deployed personnel may be exposed to toxic chemicals as a result of industrial accidents, intentional or unintentional activities of enemy or friendly forces or sabotage. Rapid risk assessment of these scenarios requires the development of new testing methods. In order to prevent serious injury to the deployed warfighter exposed to toxic substances and to minimize mission degradation due to environmentally related adverse health effects, novel human monitoring methodologies that provide near real-time detection of potential toxic injury must be developed. It is necessary to devise methodologies that will predict or identify exposure of personnel to low concentrations of harmful substances before they cause harm to an individual. It is also important to identify methodologies that are relatively non-invasive, which could include collection of urine, blood, saliva or epithelial cells from humans. Emerging biotechnologies, such as toxicogenomics, proteomics and metabonomics will be investigated for their effectiveness to identify toxic effects upon the warfighter before they can induce a reduction in health and/or operational performance or before they can induce a disease process that would not manifest for several years.

1.0 DEPLOYMENT TOXICOLOGY

Deployment of forces into unfamiliar, and potentially hostile, environments is inherently dangerous. An increasingly important issue in force protection is the toxicology associated with potential chemical and biological exposures at deployed sites. Making predictions on the probability and severity of adverse health effects from exposure to environmental contaminants at deployed sites is challenging because sufficient, accurate and reliable data are difficult, if not impossible, to obtain. The changing mission and increasing use of armed forces around the globe in non-battle operations has focused attention to threats of non-battle-related health problems (Rhomberg, 2000). Contaminated deployed environments, from theater-level combat to humanitarian missions, are becoming as hazardous as infectious disease risks in causing non-battlefield-related morbidity and mortality. Deployed personnel may be exposed to toxic chemicals as a result of industrial accidents, intentional or unintentional activities of various forces (enemy or friendly) or sabotage. Current methods of determining toxic exposures to the warfighter are not adequate to prevent serious health effects such as was induced by Agent Orange in Vietnam or to predict, prevent or rapidly rectify the scenario of the Gulf War Syndrome-like disease. The rapid risk assessment of these uncharacterized environments will require the development of new testing methodology proposed to be based on human health and mild perturbations of subclinical response. The emerging biotechnology techniques in transcription and/or protein expression (transcriptomics/genomics and proteomics), and metabonomics, involving nuclear magnetic resonance (NMR) and pattern recognition technologies, may be able to identify toxic effects occurring before they can cause any decrease in warfighter mission performance or induce a disease process that would not be manifested during duty, remain unnoticed until removal from duty, or after several years post-deployment. However, to accurately predict potential health effects to the warfighter using these emerging biotechnologies, accurate methods will be required to capture biologically relevant meaning from the generated data using these technologies. Bioinformatics, the use of computer science to obtain knowledge from biological data, is an integral part of successful methods development. Operationally, bioinformatics analysis is crucial in the collection of data and its manipulation to obtain understanding of biological processes. These technologies in combination will be used for the development of novel human monitoring methodologies that provide real or near real-time detection of potential toxic injury that will minimize mission degradation due to environmentally related adverse health effects by creating viable options for preventing or minimizing incapacitating exposures, or latent disease or disability in the months and years following deployment. A review of potential options and technologies for pre-deployment environmental surveillance, deployment exposure surveillance and post-deployment retrospective exposure surveillance has been published (Lippman, 2000).

2.0 WHAT IS UNDER OPERATIONAL TOXICOLOGY CONDITIONS?

One theory of toxicology can be perceived as being equally dependent on both time and dose. Although this concept has been around for over a hundred years, exceptions to this concept have been put forth, such as embedded in Haber's Rule (concentration (C) x duration (t) = a constant toxic effect (K)). Many toxicologists have focused on the dose-dependency of exposure, especially when effects are predominantly dose-, but not time-dependent. However, the present studies are focused on an effect at the lower portion of a response profile that would be reversible if given a modest amount of time. Most current risk predictions do not include time as a variable. While dose as a variable can be viewed as a simple function (# of molecules), time is much more complex with at least three time scales interacting with dose. The three time scales are: 1) toxicokinetic half-life (how the organism responds to a chemical), 2) toxicodynamic half-life (how is the organism affected by a chemical) and 3) frequency of exposure. According to Rozeman and Doull (1998), toxicity is defined as the accumulation of injury over short or long periods of time, which renders an organism incapable of

functioning within the limits of adaptation. This definition implies that both time and dose are functions of toxicity.

2.1 Dose-Time Relationship

The time-course for a toxicant in an organism (kinetics) is very different than the time-course of organism toxicity (dynamics). Underlying biological processes such as absorption, distribution, elimination, injury, recovery and adaptation all have different time scales. Consequences of interactions between toxicant and organism follow a causality route from toxicokinetics/toxicodynamics to manifestations of toxicity at the organism level. If recovery (adaptation, repair and reversibility) half-life exceeds the toxicokinetic half-life, then toxicodynamics becomes the rate-determining or rate-limiting factor. If the toxicokinetic half-life exceeds the recovery half-life, then toxicokinetics become the rate-determining/limiting factor. The present approach of early assessment using sensitive techniques to monitor organ-specific biomarkers and profile changes would be assessing warfighter pre-toxicology conditions in the dynamic mission environment. Because of this, baseline data and defining condition and outcome from invited survey data based on toxicodynamic organ responses and position of exposure will require secondary and tertiary follow-up sampling of the warfighter.

3.0 BIOTECHNOLOGY

It is not surprising that the word "biotechnology" connotes many definitions from the "omics" technologies to the cloning of animals. The definition of biotechnology that has been adopted by our laboratory is "the use of biological systems for the improvement of health and welfare of humans." A general perspective on biotechnology can be found in a recent publication by Frazier and Geiss (2000). Because the main focus of the present discussion pertains to novel biotechnology methods for potential monitoring of warfighter health, the derived biotechnology tools of genomics, proteomics and metabonomics will be briefly introduced.

3.1 Genomics

Major developments in large-scale genome sequencing and in the development of technology platforms to support it have had logarithmic growth over the past decade. A number of genomes have been successfully sequenced such as *Hemophilis influenzae* (Fleishman et al., 1995), yeast (Bussey et al., 1997), the nematode *Caterorhibditis elegans* (Anonymous, 1998) and recently, sequencing of the human genome. Significant portions and rough drafts of other important research organism genomes such as mouse, rat and zebra fish are presently known.

Differentially expressed genes in various types of samples (e.g. blood, target organ tissues, etc.) can be compared by conducting gene expression profiling studies. These studies provide cell/organ state information with respect to regulatory mechanisms and phenotypic activity of genes that comprise an organism's genome. Expression profiling techniques have become widely used in research and diagnostics to uncover disease pathways. DNA microarray analysis has become the most accepted technique among gene expression profiling techniques to initially monitor differential expression levels. DNA microarray analysis allows for profiling expression of tens of thousands of gene transcripts in parallel for high through-put screening, and is relatively easy to use.

Construction of gene microarrays for the quantitative assessment of transcriptional activity of tens of thousands of genes has resulted due to the availability of gene sequences and physical clones of the isolated

sequence from the coding region of these genes (Schena et al., 1995). A recent review of DNA microarray technology and its applications to mechanistic and predictive toxicology can be found in the literature (Pennie et al., 2000). We propose that the application of toxicogenomics will be highly beneficial in providing rapid profound assessment of toxicity in controlled laboratory exposure studies. Analogous to the area of drug discovery, increased mechanistic toxicity information will provide more accurate human risk assessments and direct research to biomarker pathways for identifying the best methods for monitoring human health.

3.2 Proteomics

Proteomics refer to the study of proteins expressed by an organism's genome within a certain expressed phenotype (e.g. liver cell , kidney cell, etc.). The determination of protein function is a major challenge of the post-genomic era. The speed at which target proteins can be isolated and identified will be the rate-limiting step in the establishment of proteomics as a useful diagnostic tool. The word "proteomics" was first reported in the literature by Waringer et al. (1995) and refers to the "total protein complement of a genome." Therefore, proteomics is the study of proteomes, and involves the measurement and analysis of proteins expressed by a cell at any given time. A review of proteome research can be found in the literature (Humphery-Smith et al., 1997).

While an organism's genome is relatively constant between cell types and over time, the proteome is dynamic and varies between cell types and within cells. Proteomes show a wide degree of variability in chemical characteristics such as size, solubility and concentration. A single cell contains approximately twenty thousand proteins and approximately one hundred to three hundred thousand proteins for an entire multicellular organism. These numbers are based on gene splicing prior to protein expression from the mRNA transcript, as well as from post-translational modification of the protein. Therefore, proteomics must not only concern itself with the formidable challenge of sequence diversity of proteins, but also the chemical diversity from post-translational modifications of proteins. These modifications include phosphorylation, glycosylation, acetylation, nitrosylation, proteolytic cleavage, co-factor insertion and many others, and along with up- and down-regulation of proteins are confounding factors for protein analysis.

Functional information for all cellular proteins will eventually be provided by proteomic studies. Currently, proteomic research is being driven by cell-mapping and protein expression strategies. With respect to the topic of medical surveillance technology, the protein expression strategy is the most viable. This strategy is concerned with monitoring global expression of large numbers of proteins within a cell or tissue, and quantitatively identifies pattern changes resulting from hazardous chemical or material exposures. The goal of expression profiling is to generate "protein fingerprints" that may provide insight into novel biomarkers of disease or toxicity.

3.3 Metabonomics

Metabonomics is an emerging biotechnology approach to enable high-throughput *in vivo* toxicology screening. This technology was pioneered by Jeremy Nicholson, Elaine Holmes and John Lindon at the Imperial College in London (Holmes and Shockor, 2000; Nicholson et al., 1999). A distinction must be made here before proceeding that is concerned with terminology. The quantitative measurement of the time-related multiparametric metabolic response of living organisms to pathophysiological stimuli or genetic modification is defined as metabonomics (Nicolson et al., 1999, Nicolson et al., 2002), while the assessment of metabolic composition of a cell is defined as metabolomics. Metabonomic studies investigate fixed cellular and biofluid concentrations of endogenous metabolites, as well as dynamic metabolite fluctuations, exogenous species, and molecules that arise from chemical rather than enzymatic processing (Lindon et al., 2003a). Metabonomics can be viewed as

a systems biology approach that can integrate divergent effects that occur over both time and space. Metabolomics, corresponding to the study of single cell metabolomes, can be thought of as a subset of metabonomics (Raamsdonk et al., 2001).

Metabonomics combines high resolution NMR with pattern recognition technology to rapidly evaluate the metabolic status of an animal. Using this approach, the onset, duration, severity and target organ localization can all be determined from a peripheral sample such as urine. Toxicants disrupt the normal composition and flux of endogenous biochemicals in, or through, crucial intermediary cellular metabolic pathways. These alterations may be reflected, either directly or indirectly, in the blood, which in turn may produce characteristic biomolecular traces in the urine. If a significant number of trace molecules can be monitored, the overall pattern produced may be more consistent and predictive than any single biomarker. Using high-field NMR, which is an essential component of metabonomics technology, obtaining comprehensive biochemical information is made possible. Proton (1H) NMR spectroscopy can detect all soluble proton-containing molecules with a molecular weight of 20,000 daltons or less at concentrations greater than 100 μM. The NMR spectra serve as the raw data for pattern recognition analysis, which reduces the complex multivariate data into two or three dimensions that can be readily evaluated for analysis comparisons. Acquiring this vast amount of information enables a potential for developing relatively high throughput, whole animal toxicity-screening throughout a toxic insult progression and resolution. The principles of this approach have been described (Anthony et al., 1994a; Beckwith-Hall et al., 1998; Holmes, et al., 1998a, 1992a, Holmes and Shockor, 2000; Nicholson et al., 1999). In addition, the use of NMR for metabolic analysis using biofluids has been recently reviewed (Reo, 2002; Lindon, et al., 1999). Extensive literature exists on the use of metabonomics procedures to evaluate nephrotoxicants (Robertson, et al., 2000; Holmes, et al., 1998b, 1992b; Anthony et al., 1994b) and hepatic toxicants (Robertson, et al., 2000; Beckwith-Hall et al., 1998; Holmes, et al., 1998a).

NMR analysis of biofluids (i.e. blood, urine, cerebral spinal fluid, etc) provides a window into the biochemical status of a living organism. The biofluid composition is modulated by the function of the cells responsible for its manufacture and secretion. This biochemical composition may be altered when organ damage occurs due to toxicity or disease. Such biochemical information can reflect the modes and severity of organ dysfunction. The use of NMR spectroscopy for the analyses of biofluids has recently been reviewed by Lindon, *et al.* (2003b).

NMR analysis requires minimal or no sample preparation, it is a nondestructive procedure and can usually be implemented in a noninvasive manner. Therefore, it is amenable for studies of biofluids, cell extracts, and for cell cultures and tissues *in vitro* or *in vivo*. The multinuclear capabilities of NMR provide various means to observe different chemicals. Most present published metabonomics work has exclusively used ^{1}H NMR for analyses of biofluids, but other nuclides (i.e., ^{13}C, ^{31}P, ^{15}N, ^{19}F, and ^{2}H) may provide additional information about various metabolite pools. The major limitations of the technique relate to spectral resolution and analyte sensitivity, both of which are improved by experimentation at high magnetic field strengths. Thus a majority of metabonomics studies have been conducted at $\geq$11.7 Tesla (a ^{1}H resonance frequency of 500 MHz or greater). Broadened NMR spectral lines can degrade resolution and the ability to differentiate metabolite signals. Factors influencing the NMR line-widths are due to molecular dynamics and include sample viscosity, macromolecules, binding of small molecules, compartmentaltization, and sample heterogeneity which is an inherit problem with cells and tissues.

4.0 BIOTECHNOLOGY SOLUTIONS FOR POTENTIAL MONITORING OF DEPLOYMENT HEALTH

Assessment of exposure is a critical element in risk assessment and management, and is especially important for armed forces deployed in hostile or uncharacterized sites. Enhanced capabilities that allow for real- or near real-time assessment of hazardous exposure will provide military field commanders with viable options for preventing or minimizing mission degradation due to these types of exposure, as well as preventing or minimizing latent disease or disabilities following deployment. The latter would have dire consequences on force readiness for future deployments, as well as affecting the quality-of-life of force members and their families. Therefore, monitoring hazardous exposures of deployed forces can be a valuable and cost-effective method for force protection.

Proceedings from a recent workshop entitled "Strategies to Protect the Health of Deployed U.S. Forces: Assessing Health Risks to Deployed Forces" (Rhomberg, 2000) suggests that during deployment data should be collected on personal exposures to on-site contaminants, using personal samplers and monitors, as well as the collection of exposure biomarkers whenever appropriate equipment, sampling opportunities, analytical methods and procedures are available. Furthermore, sampling strategy, because it will never be feasible to monitor all personnel, and documentation of force descriptors (e.g. age, ethnicity, genetic susceptibility, prior exposure and medical histories and other stress factors) cannot be overlooked.

Assessment of deployed armed forces for health effects resulting from exposure to unknown chemical and material hazards is challenging, particularly when the personnel being monitored serve as the biological monitors. One of the first challenges that is immediately apparent is the limited access to sources of biological material to be sampled. Biological samples collected from the warfighter must be obtained by non-invasive means when possible (e.g. urine, saliva, etc.), and only by minimally-invasive methods in all other cases (e.g. blood, spinal fluid, etc). Therefore, biological markers of exposure must be present in these types of biological materials if they are to be useful for monitoring human health effects due to hazardous exposures. Biomarker analysis will usually be performed on components of blood or urine due to sufficient quantities and relative ease of collection. Presented below are two examples from our laboratory using genomics and metabonomics technologies for potential toxicity assessments in rats exposed to a model liver toxicant. We are currently in the process of establishing proteomic capabilities for toxicity assessments.

4.1 Genomic Assessment of Toxicity Using Peripheral Blood Mononuclear Cells (PBMC)

The peripheral blood lymphocyte can be used for toxicogenomics assessment of hazardous exposures. Because these cells contain genetic material and circulate throughout the body, they can interact with toxic chemical (or metabolites there of) or biological agents. Lymphocytes have been shown to integrate exposure over extended time intervals because they are long-lived (Braselmann et al., 1994) and do not divide *in vivo*. A molecular-based approach such as genomics has the potential to be a highly sensitive technology for monitoring chemical exposure. Employing a highly parallel technique such as GeneChip analysis will enhance the probability of identifying biomarkers for chemical exposure.

A preliminary study was conducted in our laboratory to investigate whether PBMC could be used as a surrogate tissue for genomic monitoring of hazardous chemical exposures. A general overview of this genomic approach is shown in Figure 1. The objective of this study was to identify differential gene expression in PBMCs following exposure to a known liver toxicant (alpha-naphthylisothiocyanate; ANIT), and to identify variables useful for classification for pattern recognition techniques. PBMCs from Fisher 344

rats (3 rats/dose) were analyzed by transcriptomic profiling on an Affymetrix RAE230A GeneChip (~16,000 probe sets/chip) using an Affymetrix technology platform. Discriminant analysis and classification variable selection of the data were performed using various statistical algorithm software packages (Matlab, Insightful, Partek Pro, etc.).

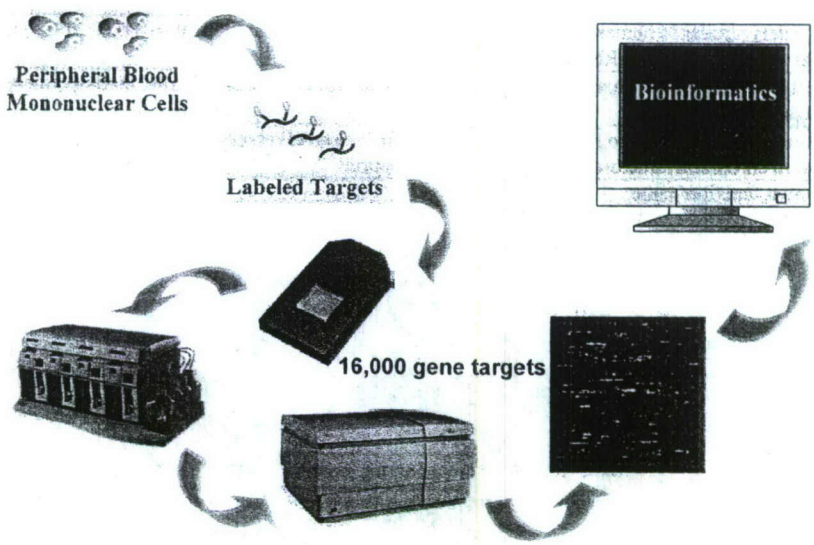


Figure 1: General overview of genomic assessment methodology for toxicity screening using peripheral blood mononuclear cells (PBMC) as a surrogate tissue

Changes in gene expression in rat PBMC following ANIT treatment (0.5, 1.0, 5.0 and 10.0 mg/kg) were analyzed using a t-test ($p< 0.05$), as well as the Affymetrix Comparison Analysis (Wilcoxon Signed Rank test, $p < 0.005$). To minimize potential false positives, a "double-double" approach was used to increase the stringency of the analysis (i.e. the differences in gene expression between the treated and untreated groups have to be significant in both the t-test and the Affymetrix Comparison Analysis in at least two doses). Thirty-two genes were identified that met this criterion, and these were then clustered into three groups using a correlation coefficient clustering technique based on their response to the ANIT treatment. Of these 32 genes, 14 showed significant changes at the lowest ANIT dose tested (Table 1). The clustering pattern of these genes is shown in Figure 2.

Examination of the 32 genes in these three clusters reveals that: 1) the genes in Cluster 1 are involved in biological processes specific for cell growth and maintenance including chromatin structure/dynamics, chromosome partitioning, transcription and translation, 2) the genes in Cluster 2 are involved in cell adhesion, immune response and protein synthesis/degradation and 3) the genes in Cluster 3 are involved in cell adhesion, chemotaxis, cytoskeleton, RNA splicing, protein synthesis/folding/degradation and metabolism of lipid and nucleotides. Interestingly, all nine genes in Cluster 1 showed significant changes at the lowest ANIT dose tested (0.5 mg/kg), while only 3 and 2 genes were significant changed in Cluster 2 and Cluster 3, respectively (see Table 1).

Table 1: Changes in Gene Expression in Rat PBMC Following 0.5 mg/kg ANIT Treatment

GENE CLUSTER 1
Catalase
Embigin, belongs to immunoglobulin super family; putative cell adhesion molecule
Pituitary tumor transforming gene; may play a role in cell growth and/or maintenance
Lamin A; nuclear envelope protein lamin, intermediate filament superfamily; involved in cell cycle/cell division, chromosome partitioning and nuclear structure
EST, moderately similar to human multisynthetase complex auxiliary component p18 (MCA3); a component of bifunctional and monospecific tRNA synthetase complex
EST, with strong similarity to human yippee protein; a novel family of putative zinc binding proteins highly conserved among eukaryotes
EST, with moderate similarity to human sirtuin 2, isoform 1; a novel receptor with seven transmembrane domains; may be involved in chromatin structure/dynamics and transcription
EST, unknown function/name
EST, unknown function/name

GENE CLUSTER 2
Allograft inflammatory factor-1 (AIF-1); expressed in inflammatory cells; may play a role in macrophage activation
Bst1: Bone marrow stromal cell antigen 1
Ly6 homolog RK3 precursor; superfamily: Ly-6 antigen; Ly-6 homology; GPI-anchored protein
Fc receptor, IgG, low affinity III; isoforms differ in IgG subclass-binding specificity; IgG Fc receptor of natural killer cells and macrophages
Ficolin 2 precursor (Collagen/fibrinogen domain-containing protein 2)
96KDa lysosomal membrane sialoglycoprotein precursor (lysosomal-associated membrane protein 2)
Cathepsin H, lysosomal proteinase; belongs to peptidase family C1.
60S Ribosomal Protein L21
EST, similar to goliath-related E3 ubiquitin ligase 4; may regulate growth factor withdrawal-induced apoptosis of myeloid precursor cells

GENE CLUSTER 3
Ferritin, heavy polypeptide 1; Ferritin subunit H
EST, moderately similar to mouse lymphocyte antigen LY-6E precursor; attached to the membrane by a GPI-anchor
Chemokine-like factor isoform a; chemoattractant for neutrophils, monocytes and lymphocytes; may play important roles in inflammation
Chemokine-like factor isoform a; chemoattractant for neutrophils, monocytes and lymphocytes; may play important roles in inflammation
Proteasome ring12 chain; superfamily: multicatalytic endopeptidase complex chain C5
SH-PTP2 protein tyrosine phosphatase, non-receptor type 11; Chaperonin subunit 4 (delta): molecular chaperone t-complex-type superfamily
Splicing factor, arginine/serine-rich 5; play a role in pre-mRNA splicing: may be required for cell growth/cell cycle progression
EST, similar to human splicing factor, arginine/serine-rich 4 isoform a
Beta-actin (cytoplasmic)
ESTs, highly similar to human AR16, actin-related protein 2/3 (ARP2/3) complex subunit 5 (16 kDa subunit)
Diphosphoinositol polyphosphate phosphohydolase type II
Nucleoside diphosphate kinase
Phosphoglycerate kinase; phosphoglycerate kinase superfamily
EST, unknown function/name

Significant at $p < 0.05$

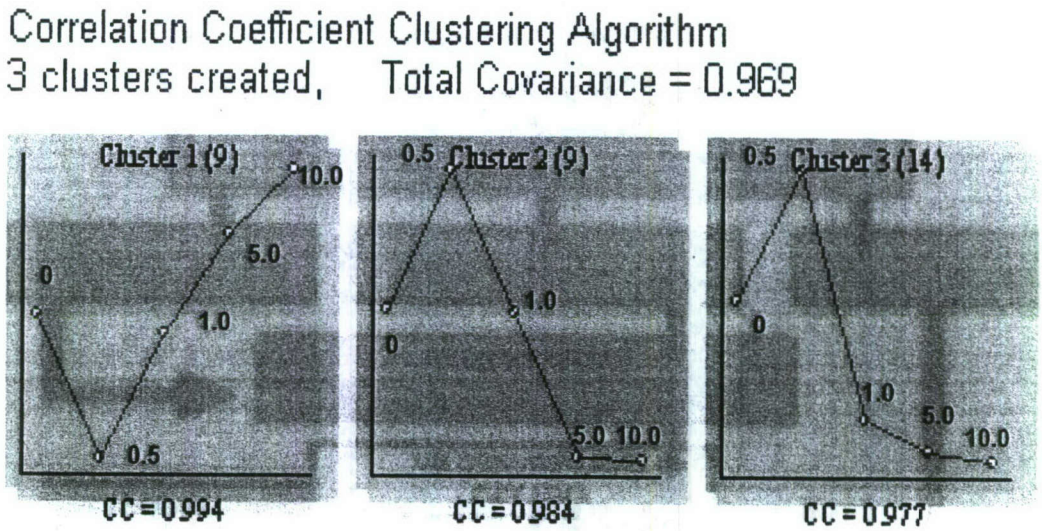


Figure 2: Clustering analysis of 32 genes with significant changes in expression. Graphs represent the dose response of these genes to ANIT treatment (0.5-10.0 mg/kg) when analyzed using correlation coefficient clustering technique. Three clusters, one up-regulated and two down-regulated, were observed.

4.2 Metabonomic Assessment of Toxicity Using Urine

Metabonomics is an approach used to characterize the metabolic profile of a specific tissue or biofluid. Because many biofluids can be easily obtained either non-invasively (urine) or minimally invasively (blood), they are typically used in metabonomic studies. However, other *in vivo* biofluids such as saliva, cerebrospinal fluid, bile, and seminal fluid, as well as *in vitro* biofluids such as cell culture supernatants and tissue extracts, can also be used. Metabonomics is an attractive approach to the study of time-related quantitative multivariate metabolic responses to pathophysiological processes because biological and chemical agents, or drugs, cause perturbations in the concentrations and fluxes of endogenous metabolites involved in critical cellular pathways. In other words, cells respond to toxic insult or other stressors by altering their intra and/or extracellular environment in an attempt to maintain a homeostatic intracellular environment. This metabolic alteration is expressed as a "fingerprint" of biochemical perturbations that is characteristic of the type and target of a toxic insult or disease process. These metabolic alterations are often seen in the urine as changes in metabolic profile in response to toxicity or disease as the body attempts to maintain homeostasis by eliminating substances from the body. Subtle responses to toxicity or disease under conditions of homeostasis also result in altered biofluid composition.

A study was conducted in our laboratory to investigate whether metabonomics could be used as an approach to monitor hazardous exposures to deployed forces. A general overview of our metabonomic experimental design is shown in Figure 3. The objective of this study was to develop NMR monitoring and pattern recognition technologies to screen urine/blood of deployed personnel to identify potential target organ toxicity resulting from low-level exposure to deployment-related chemicals. Using a Varian 600 mHz NMR

instrument, 24 h rat urines were collected daily over four days from animals exposed to a range of ANIT concentrations and analyzed. Pre- and post-exposure blood samples were also collected for clinical chemistry measurements, and tissue samples were processed for histopathological evaluations.

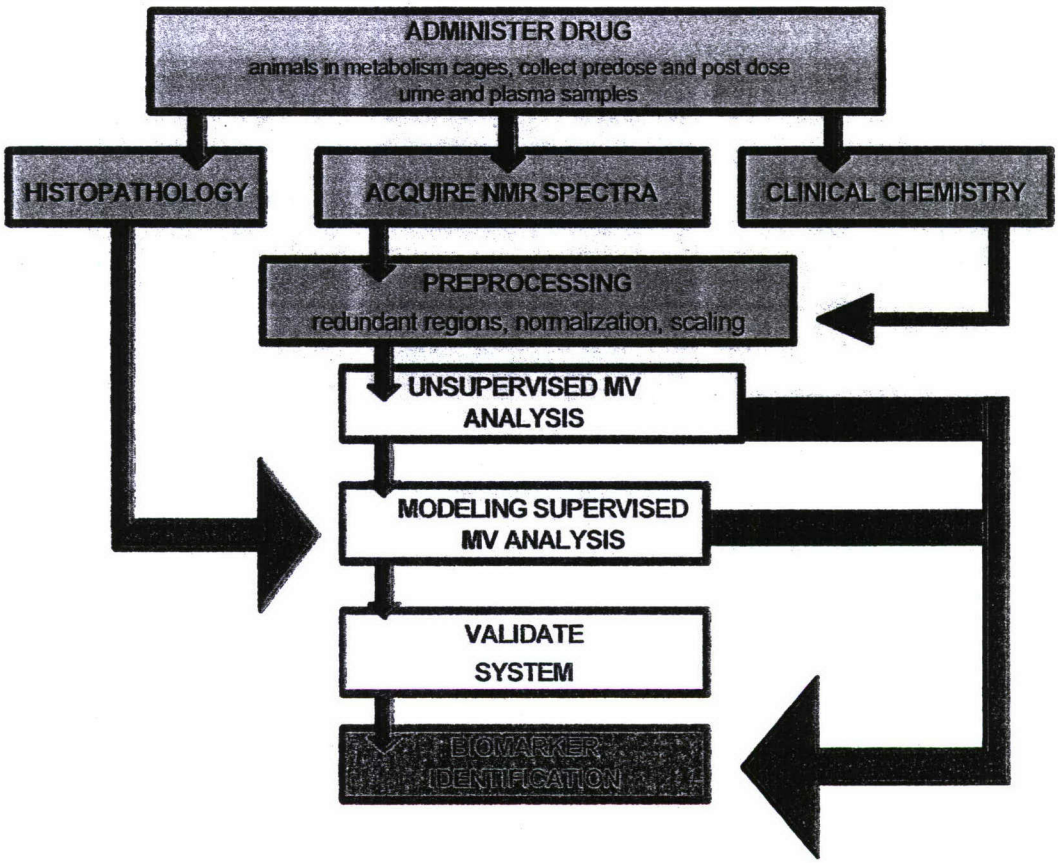


Figure 3: Overview of metabonomics study experimental design

Clinical chemistry analysis results from this study indicated that rats exposed to 100 mg/kg ANIT showed significant elevation of liver enzymes throughout the 4-day study period when compared to control (Table 2). Rats exposed to 50 mg/kg ANIT indicated significant elevation in liver enzymes following 24 h post-exposure that appeared to recover to normal levels by the end of the study at day 4. Rats exposed to ANIT concentrations less than 50 mg/kg did not demonstrate any significant change in liver enzymes throughout the study. The highest ANIT dose tested that did not induce any clinical effects on the liver was 20 mg/kg.

Table 2: Effect of α-Naphthylisothiocyanate (ANIT) Hepatotoxin on Selected Liver Clinical Chemistry Parameters (Mean ± SD)

	ALKP		ALT		AST	
Treatment	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4
Control (Corn Oil)	280± 54	288± 25	38± 8	40± 11	82± 18	88± 18
ANIT (100 mg/kg)	370± 22	609± 155	152± 60	125± 37	278± 142	229± 101
ANIT (50 mg/kg)	380± 115	397± 50	232± 154	56± 16	552± 98	73± 13
ANIT (20 mg/kg)	259	335± 103	35	41± 19	91	72± 26
ANIT (10 mg/kg)	291± 20	332± 127	38± 6	36± 9	79± 10	88± 16
ANIT (1 mg/kg)	315± 22	293± 16	41± 11	39± 6	88± 23	68± 3
ANIT (0.5 mg/kg)	314	358± 129	43	29± 26	111	64± 16
ANIT (0.1 mg/kg)	-----	331± 163	-----	33± 6	-----	76± 9

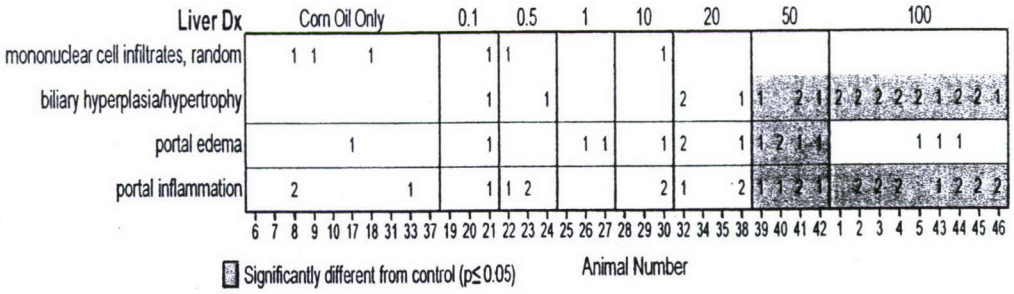
(Mean ± SD) Significantly different than control ($p < 0.05$). ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase

alterations in the NMR spectra, with effects on citrate predominating (Figure 4, Panel A). These alterations in urine profile were found to occur 24 h following expose and became more pronounced at 48 h post-exposure. In contrast, rat urine collected from animals exposed to 10 mg/kg ANIT; a dose that did not indicate any significant clinical effects over 4 days, indicated an altered NMR urine profile following 24 h post-exposure that was reversing back to normal at 48 h post-exposure (Figure 4, Panel B). Results to-date indicated that NMR analysis could identify altered urine profiles from rats exposed to the lowest ANIT concentration tested (1.0 mg/kg; data not shown).

5.0 CONCLUSION

Preliminary findings from our laboratory using genomics and metabonomic technologies indicated that both approaches appear to possess sensitive differential indicators of low-level chemical exposure. These approaches were able to detect changes in biofluid profiles (blood and urine) from animals exposed to the liver toxicant ANIT at doses well below that causing clinical effect. However, the sensitivities of these biotechnology approaches can be viewed as a double-edged sword. On the one hand, these technologies are very sensitive and give an early indication of exposure; while on the other hand, mission capability may be adversely impacted because field commanders constantly have to make decisions concerning personnel health status for assignments. Therefore, it is imperative that field commanders only are required to make this kind of decision when there are valid concerns of potentially hazardous exposures. Unquestionably, the detection of toxic insult by means of biochemical effects is difficult near the toxic threshold, yet these are frequently the most important effects to elucidate. One of the goals of our "omics" research is to identify significant alterations in biofluid profiles that occur near the threshold of toxicity for a particular target organ tissue that can serve as a biomarker of an exposure of concern. It is highly unlikely that a single "omic" technology will become a panacea for all future chemical toxicity testing studies. However, it is possible to use these biotechnologies in an integrated approach to explore the relationships between biofluid (or tissue) pattern profiles and toxicity that will lead to the generation of novel biomarkers of toxicity, and genetic identification of sensitive populations.

Table 3: Histopathology Evaluation of Rat Livers from Animals Exposed to Varying Concentrations of ANIT



Note: Histopathology severity score (no value = no lesions, 1=minimal, 2=mild, 3=moderate, 4=marked, 5=severe). Dose above each non-control column is in mg/kg. Significance is based on a 2-tailed Wilcoxon Rank Sum test.

At termination of the ANIT study (day-4), rats were euthanized and liver samples were fixed in 10% formalin. These samples were then processed for light microscopic evaluation. Liver tissues were also stained with hematoxylin and eosin prior to evaluation. Histopathology examination of the liver indicated that the severity score never exceeded "mild" in any of the animals exposed to ANIT. However, significant changes were only observed in rats exposed to ANIT at 50 mg/kg or higher (Table 3). The observed liver histopathology was consistent with effects known to occur in liver following exposure to the model choleostatic liver toxicant ANIT.

Rat urine was collected daily, beginning 24 h prior to exposure, over the course of the 4-day study from both control and ANIT treatment groups into cups containing 1.0 mL of 1% sodium azide maintained at 0°C. Rat urine collected at the end of each 24 h period was flash-frozen in liquid nitrogen and stored at minus 20°C prior to analysis. Rat urine sample were analyzed using a Varian 600 mHz NMR instrument. Preliminary results of NMR analysis of urine collected from rats exposed to 100 mg/kg ANIT identified a number of

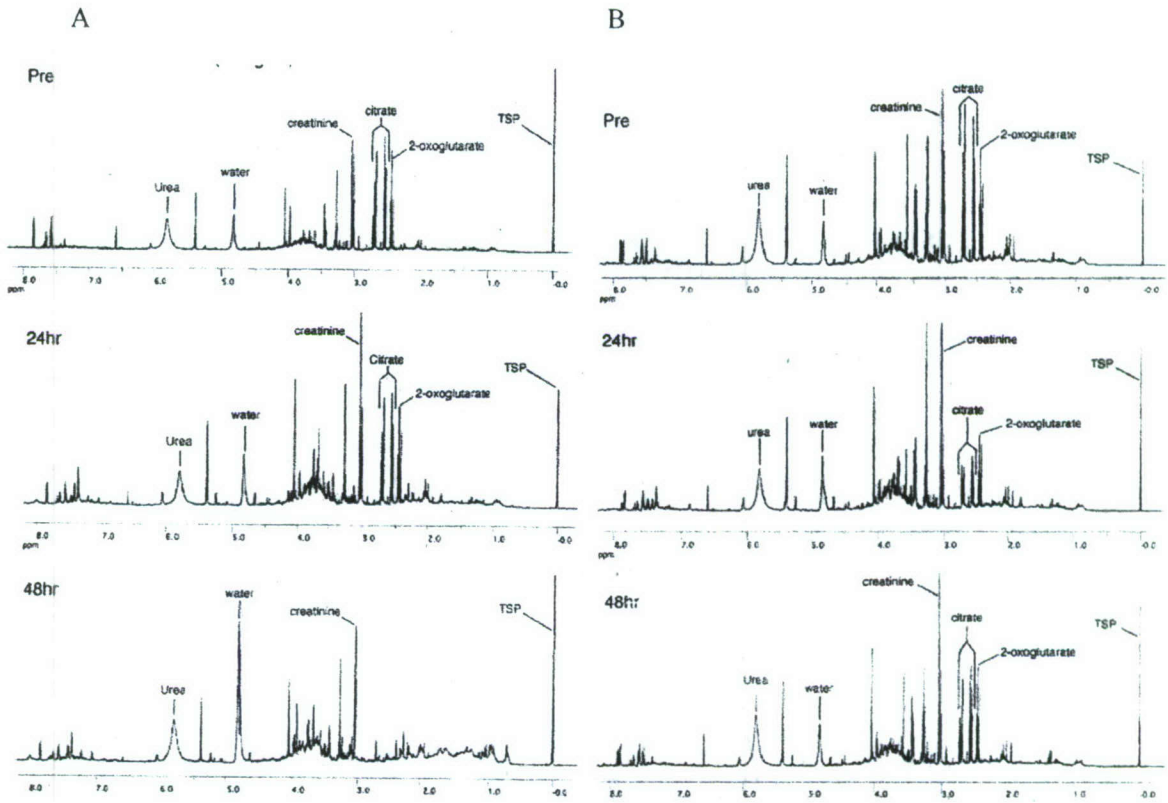


Figure 4: NMR spectra (600 mHz) of rat urine from rats exposed to ANIT at 24 h pre-exposure, and 24 h and 48 h post-exposure. A; 100 mg/kg ANIT. B; 10 mg/kg ANIT Note reduction in citrate peaks over time in the 100 mg/kg treatment group (Panel A) and the recovery of these peaks in the 10 mg/kg treatment group (Panel B) at 48 h-post exposure.

Applying any of the "omics" technologies will require generating databases for control animals and humans, disease states, and animals used in drug and toxicity testing. In addition to database generation, bioinformatic tools will need to be developed to statistically analyze the large volume of complex multivariate data generated by these technologies. Therefore, much work remains to be accomplished before these technologies can be used to provide biologically relevant predictions of hazardous risk due to chemical or material exposure. However, these technologies will eventually provide a global view of a complex organism's response to physiological stressors. This will lead to a better understanding of the relationship between gene function and metabolism in health and disease.

6.0 ACKNOWLEDGEMENTS

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SYMPOSIA DISCUSSION - PAPER 19

Authors Name: Dr Del Raso (US)

Discussor's Name: LtCol Saracli (TU)

Question:
How does chronic exposure to toxic chemicals affect the diagnostic value of the system, especially for soldiers having possible chronic exposure history, previously?

Author's Reply:
It is envisioned that a metabonomics (e.g. NMR analysis of bio fluids) will be utilized to monitor military personnel for hazardous chemical exposure. This analysis would be performed pre -, during and post-deployment. Pre-screening will undoubtably require a questionnaire to determine pre-deployment status (ie.smoker, alcohol, diet, previous known exposures). If a person is deemed health but has a documented chronic exposure history, that person's urine profile pre-deployment would be reflected. If this base-line profile is significantly altered during post-deployment, it would be identified upon subsequent urine analysis. This assessment would be based on the assumption that all mechanisms-of-action per target organ have been characterized. This would result in the establishment of benchmark urine profile levels for each target-organ mechanism of toxicity that exceeds established benchmark. Therefore, unless chronic exposure results in the urine profile for a particular target organ mechanism of toxicity (that exceeds an established bench mark), it will not flag or be of concern.